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13. ABSTRACT (Maximum 200 Words)

Prostate cancer is the second leading cause of cancer death in men in the United States. A few epidemiology studies have indicated that exposure to PhIP, a rodent prostate carcinogen formed in meat during cooking, may be an important risk factor for prostate cancer in humans. Therefore, a highly sensitive biomarker assay is urgently needed to clarify the role of PhIp in prostate cancer. The goal of this project is to develop an assay that can be used to more accurately quantify human exposure to PhIP and potential prostate cancer risk. Our hypothesis is that an Accelerator Mass Spectrometry-based method can be developed to measure protein adducts of PhIP in the blood of humans. This will provide a measure of the internal dose, as well as the capacity for carcinogen bioactivation to a form that can initiate the cancer process. Towards this goal, we have characterized an adduct formed by PhIP in vitro with the amino acid cysteine. This adduct should provide a biomarker of dietary PhIP exposure and potential prostate cancer risk that could be used to identify individuals for prevention and for monitoring the effect of chemoprevention strategies.

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Introduction:

Prostate cancer is the second leading cause of cancer death in men in the United States, with African-Americans having the highest rate of prostate cancer in the world. Despite these statistics, the specific causes and risk factors for prostate cancer and reasons for the racial disparity remain elusive. A few epidemiology studies have indicated that exposure to PhIP, a rodent prostate carcinogen formed in meat during cooking, may be an important risk factor for prostate cancer in humans. There is also some evidence that African-American children have a 2-3 fold higher exposure to PhIP than White American children. Children are an important population to study because carcinogen exposure during development may lead to increased prostate cancer risk in later life. However, the epidemiology data are based upon PhIP exposure estimates from dietary questionnaires, rather than measurement of molecular markers that more accurately quantify an individual's internal dose and potential cancer risk. Therefore, a highly sensitive biomarker assay is urgently needed to clarify the role of PhIP in prostate cancer.

The goal of this project is to develop an assay that can be used to more accurately quantify human exposure to PhIP and potential prostate cancer risk. Our hypothesis is that an Accelerator Mass Spectrometry-based method can be developed to measure protein adducts of PhIP in the blood of humans. This will provide a measure of the internal dose, as well as the capacity for carcinogen bioactivation to a form that can initiate the cancer process. In a proof-of-principle study, we will use the assay to investigate the hypothesis that African-Americans may be at greater risk for prostate cancer than Whites because they have adduct levels that are 2-3 fold higher in childhood.

Our aims are to 1) Characterize the protein adducts formed by PhIP with the blood protein albumin. 2) Develop an ultrasensitive radiommunoassay for PhIP albumin adducts so that they can be assessed in populations of people. 3) Measure PhIP-albumin adduct levels in blood samples obtained from African-American and White male children.

Body:

During the first year of this grant (February 1, 2003 to January 31, 2004) we have made significant progress in specific aim 1 of the proposal. The progress is described as follows:

Specific aim #1: Characterize the adducts formed with the blood protein albumin after exposure to PhIP.

The goal of this aim is to use *in vitro* methods to synthesize sufficient quantities of adducts for characterization by mass spectrometry and then establish if these adducts are formed *in vivo* in an animal model and humans.

Towards this goal, we have characterized a peptide adduct formed by the putative genotoxic metabolite, *N*-acetoxy-PhIP. A model peptide with the internal sequence Leu-

Gln-Lys-Cys-Pro-Tyr, which is homologous to a potential target sequence for heterocyclic amines such as PhIP in human serum albumin, was reacted with *N*-acetoxy-PhIP and an adduct was identified and further characterized by LC-ESI-MS/MS. *N*-acetoxy-PhIP was covalently bound to the peptide via cysteine and the exocyclic amino group of PhIP. This work has been accepted for publication in Food and Chemical Toxicology (Chepanoske *et al.*, see appendix for the manuscript).

The cysteine adduct was also formed when human serum albumin was reacted with N-acetoxy PhIP, as previously described (Brown *et al.* 2001, and Chepanoske *et al.* In press). To remove non-covalently bound or unreacted N-acetoxy PhIP, the reacted samples were purified using affi-gel columns and then concentrated using MiliQ centricons. The albumin samples were then digested by trypsin and Glu-C to allow analysis by mass spectrometry. Samples were desalted using zip tips C18 and/or C18 Sep Pak cartridges upon injection into mass spectrometer. Data were collected on an ICR-FT mass spectrometer (Bruker, CA) operating in the positive mode. Each data set consisted of 400 experiments of 8-16 scans/experiment with a delay time of 0.5 sec. PhIP adducts seen in the Glu-C digest were confirmed in the tryptic digest and were all with cysteines on the surface of the protein.

Towards establishing if the adduct is formed *in vivo*, albumin was isolated from human and rat plasma samples using previously published methods (Dingley *et al.*, 1998). We used a pooled plasma sample from rats that had been dosed with [^{14}C]PhIP at a dose of 90 μg/kg body-weight. The human sample was obtained from a previous study in which volunteers were administered 1 μg/kg body-weight [^{14}C]PhIP (Dingley *et al.*, 1999). Heterocyclic amine adducts with a sulfur linkage to cysteine are cleaved by acid hydrolysis (Turesky *et al.*, 1987). Hence by treating the albumin samples with acid, recovering PhIP through organic extraction and then analyzing using accelerator mass spectrometry, we can quantify the amount of the cysteine adduct formed in the rat and human plasma samples.

The acid hydrolysis method of Magagnotti *et al.* 2000 was used, with a few minor modifications. 500 μg of rat albumin or up to 1mg of human albumin in 1-2ml buffer in 15ml glass tubes were acidified by adding 220 μl of 1N HCl, and hydrolyzed at 80°C for 1hr. Immediately following the hydrolysis, the pH of the solution was made basic by adding 30 μl of 10N NaOH, and hydrolyzed PhIP was extracted three times with 1.5 times the sample volume of ethyl acetate. Extracted samples were dried by SpeedVac and analyzed by AMS. Preliminary data showed that 17% and 24% of the [^{14}C]PhIP in the rat and human samples, respectively, was recovered following acid hydrolysis. Therefore, this result implies that the cysteine adduct represents 17% of the total covalently bound PhIP in rats and 24% of the total covalently-bound PhIP in humans. This experiment will be repeated to verify the finding and to establish the variability between individuals.

Key Research Accomplishments:

During the first year of this grant, we have shown that:

- N-acetoxy PhIP, a bioactive form of the prostate carcinogen PhIP, forms a protein adduct with cysteine in a model peptide *in vitro*. This finding is being published in a peer-reviewed journal.
- The cysteine adduct is also found in albumin that has been reacted with N-acetoxy PhIP. The adducts form on the surface of the protein.
- Preliminary data indicates that the cysteine adduct is formed *in vivo* in rats and humans exposed to PhIP.

Reportable Outcomes:

Publications

Cindy Lou Chepanoske, Karen Brown, Kenneth W. Turteltaub, and Karen H. Dingley. Characterization of a peptide adduct formed by *N*-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a reactive intermediate of the food carcinogen PhIP. In Press, *Food and Chemical Toxicology*.

Employment/Research Opportunities

Over the period of this grant, we were able to hire a post-doctoral fellow (Anh-Tuyet Tran), a biomedical scientist (Sylvia Ahn) and a summer student, Kristin Stoker from UC Berkeley, to work on this project. This was the first opportunity for Ahn and Sylvia to work in prostate cancer research.

Conclusions:

During the first year of this grant, we have made significant progress towards our specific aims. We have shown that a reactive form of PhIP forms adducts with proteins *in vitro* and have characterized an adduct with cysteine. This adduct is formed with albumin *in vitro* and preliminary data indicates that it is also formed *in vivo* in rats and humans. We will now attempt to synthesize sufficient quantities of the adduct to raise antibodies. The antibodies will then be used in an immunoassay to quantify adduct levels in human blood samples.

"So What?"

As a result of the work completed over the past 1 year, we have established the structure of an albumin adduct formed by a prostate carcinogen in blood. This will be used to provide a biomarker of dietary PhIP exposure and potential prostate cancer risk that could be used to identify individuals for prevention and for monitoring the effect of chemoprevention strategies.

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Appendices:

Copy of manuscript entitled:

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Characterization of a peptide adduct formed by *N*-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a reactive intermediate of the food carcinogen PhIP

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Abbreviations:

PhIP:	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
HCA:	heterocyclic amine
LC-ESI-MS	liquid chromatography-electrospray ionization mass spectrometry
MS/MS	tandem mass spectrometry
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
HPLC	high performance liquid chromatography
m/z	mass-to-charge ratio

Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a member of a class of compounds known as the heterocyclic amines (HCAs) that are formed in meat during cooking. It is a multi-organ carcinogen in rodents and forms adducts and with DNA and protein. Although protein adducts are not thought to be involved in cancer development, they may be useful as internal dosimeters of PhIP exposure and bioactivation. Towards the goals of characterizing the adducts formed in humans and the development of an assay for quantitation of adduct levels, we have characterized a peptide adduct formed by the putative genotoxic metabolite, *N*-acetoxy-PhIP. A model peptide with the internal sequence Leu-Gln-Lys-Cys-Pro-Tyr, which is homologous to a potential target sequence for HCAs in human serum albumin, was reacted with *N*-acetoxy-PhIP and an adduct was identified and further characterized by LC-ESI-MS/MS. *N*-acetoxy-PhIP is covalently bound to the peptide via cysteine and the exocyclic amino group of PhIP. Future work is needed to establish if this adduct is formed and is stable *in vivo* in humans following exposure to PhIP.

Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a heterocyclic amine (HCA) which is formed in relatively high concentrations during cooking of meat (Layton 1995). PhIP is a potent mutagen in bacteria and mammalian cell genotoxicity assays (Thompson 1987) and has been shown to cause tumors in the colon, prostate, and breast following high dose exposures in rats (Ito 1991; Shirai 1997). Indeed, some epidemiology studies have linked dietary HCA exposure to cancer in these organs in humans (reviewed by Sinha 2002).

Metabolic activation of PhIP, thought to be mediated primarily through cytochrome P4501A2, followed by *N*-acetyltransferase and sulfotransferase activation (Buonarati 1990; Boobis 1994; Turesky 1998), forms reactive intermediates that can form covalent adducts with DNA and protein (Figure 1). Protein adducts are formed after exposure to chemical carcinogens and provide a quantitative measure of the internal dose, as well as an individual's capacity for carcinogen bioactivation (Skipper 1994). Thus, measuring protein adducts may provide an indication of the potential risk of cancer following exposure.

PhIP has been shown to bind to the blood protein albumin in humans and the binding is relatively stable (Dingley, 1999). However, although protein adducts of PhIP are likely to provide an important biomarker of individual exposure and metabolic capacity, there has been very little and incomplete PhIP-adduct characterization. A protein adduct of the HCA 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) has been fully characterized and forms a cysteine adduct with albumin via a sulfinamide linkage (Turesky, 1987). However, an analogous adduct was not identified from *in vitro* reactions

between the reactive metabolite *N*-acetoxy-PhIP and glutathione or cysteine. Instead, *N*-acetoxy-PhIP formed unstable conjugates. These conjugates were not fully characterized, but high resolution mass determination indicated that the 2-amino group of PhIP may have been lost and replaced by cysteine in a C-S linkage (Reistad, 1994).

Our aim was to identify and more fully characterize a protein adduct formed by PhIP, which could be used as a biomarker of PhIP exposure and bioactivation. This will be of great use in molecular epidemiology studies aimed at understanding the role of PhIP in cancer development. Towards this goal, we have characterized a peptide adduct formed by the putative genotoxic metabolite, *N*-acetoxy-PhIP. Previous results from our laboratory have shown that PhIP binds to albumin in the blood of humans (Dingley 1999), however the large amount of non-adducted protein and subsequent digested peptides would possibly hinder the initial characterization of these protein adducts by traditional mass spectrometry techniques without prior enrichment. Therefore, a model system containing a peptide with one each of the 20 common amino acids and an internal sequence identical to rat and homologous to human albumin, Leu-Gln-Lys-Cys-Pro-Tyr, was chosen for the initial analysis. This homologous sequence also represents the only cysteine in human and rat albumin that is not involved in disulfide bridges within the protein, and its slightly exposed nature and nucleophilic character make it a likely candidate for protein binding (Sugio 1999). Additionally, previous results have shown the formation of protein adducts via cysteine from exposure to IQ and PhIP (Turesky 1987, Reistad, 1994).

Materials and Methods

Materials. The peptide VSATWHLQKCPYERMDFNIG was synthesized and purified commercially (SynPep Corporation, Dublin CA). *N*-acetoxy-PhIP was synthesized and purified as described previously (Brown 2001). HPLC grade solvents and all other chemicals were purchased from Aldrich (St. Louis, MO).

Covalent adduct formation. Two hundred micrograms of the model peptide was reacted with an equimolar amount of *N*-acetoxy-PhIP in potassium phosphate buffer, pH 7, with a total reaction volume of 1 mL. *N*-acetoxy-PhIP was dissolved in methanol just prior to use and was added to the reaction in 3 aliquots over 60 min. The reaction was stirred at 37 °C for 60 min and then the sample was dried and redissolved in 100 µl 10% acetonitrile containing 0.1% formic acid. The reactions with subsequent LC-MS/MS analyses were repeated in triplicate to insure reproducibility of the results. LC-MS/MS was also repeated after storing the reaction for 5 days at room temperature.

HPLC methods. A microbore HPLC unit equipped with an autoinjector (Shimadzu Scientific, Inc., Columbia, MD) was used as the inlet for the mass spectrometer. Without prior cleanup, 5 µl of the sample was loaded onto a 1.0 mm ID x 250 mm C_{18} column (Vydac 238MS51, Hesperia, CA) equilibrated in water with 0.1% formic acid (Solvent A) and 15 % acetonitrile with 0.08% formic acid (Solvent B) at 50 µl/min. The mixture was eluted using a gradient of 40% B in 50 min.

Mass spectrometry. An LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) operating in the positive mode was used. The ionization source was operated under normal spray conditions with total flow rates of 50 µl/min and a sheath gas flow of 60 psi. Typical conditions included a spray voltage of approximately 5 kV and a

capillary temperature of 250-270°C. Specific focus parameters included 21 V to the heating capillary and –20 V to the tube lens. ESI(+)MS/MS was carried out using the following conditions: isolation width was 1.2 m/z, activation amplitude was 28%. Activation time was 60 ms, and the activation Q was 0.27. For third-stage MS experiments, the activation amplitude was 32% and all other parameters remained the same. Xcalibur software was used to control the mass spectrometer and the HPLC as well as analyze the data. Protein Prospector Internet-based software (UCSF) was used to predict ion fragmentation.

Results and Discussion

The covalent species formed during the reaction of *N*-acetoxy-PhIP and a model peptide *in vitro* was identified using microbore HPLC coupled to an LCQ ion trap mass spectrometer. The control reactions (peptide alone and *N*-acetoxy-PhIP alone) were also monitored using LC-MS (Figure 2). The results of the control experiments illustrate a single peak in the base peak chromatogram for the peptide and an unresolved broad peak in the base peak chromatogram for the *N*-acetoxy-PhIP control. The spectrum revealed that the starting material without the presence of a biomolecule quickly converts into *N*-hydroxy-PhIP; the expected *m/z* 283.1 of *N*-acetoxy-PhIP could not be extracted from the chromatogram. Conversely, the peptide is quite stable in the reaction conditions. The spectrum revealed that the peak contained a majority of the 20-mer peptide, *m/z* 1198.5, and a small amount of degradation product that was identified as the peptide with a loss of 2 amino acids and water at the C-terminus. In the reaction of *N*-acetoxy-PhIP and the model peptide, the chromatogram revealed an adduct species with a *m/z* 1309.5,

corresponding to the doubly charged ion of the peptide mass with a covalent modification by PhIP, indicated by an increase in mass by 222.0 Da (Figure 3). Importantly, the adduct was still present after storage at 25°C for 5 days. This apparent stability is in agreement with previous work in our laboratory that showed the formation of stable adducts *in vivo* measured in human plasma after the administration of PhIP (Dingley et al.,1999).

As illustrated in Figure 3, there are a number of unidentified peaks in the base peak chromatogram. Even though the chromatogram indicates that the covalent species is a minor product, it was possible to manually extract 1309.5 as a predicted adduct species from the chromatogram; this species tailed significantly after the elution of the unreacted peptide. However, the other peaks did not correspond to any predicted side products in the reaction such as additional covalent adducts to the peptide or N-acetoxy-PhIP by-products. It was predicted that the N-acetoxy-PhIP by-products would be prominent in the reaction monitoring, so we scanned a range from 400 to 1600 *m/z*. Indeed, the peak at 1309.5 was the only peak we could discern resulting from the reaction of N-acetoxy-PhIP and the peptide. Other predicted molecular weights of adducts could not be extracted from the chromatogram. Presumably the unidentified peaks are derived from the high molecular peptide, as the protonated PhIP $[M+H]^+$ at m/z 225 and by-products would be below mass m/z 500.

The initial MS analysis and the molecular weight of the adduct species indicates a nucleophile displacing the acetyl group and possibly suggests a linkage via the exocyclic amino group of *N*-acetoxy-PhIP. Relative to the peak intensity of the unreacted peptide in the mixture, it is predicted that roughly ~15 % of the adduct was formed. This is an

approximate value since there wasn't a calibration curve constructed for the unreacted peptide.

The adduct species was further characterized by LC-ESI(+)-MS/MS to determine the exact amino acid containing the modification. Although hypothesized to be cysteine, other amino acids such as lysine or arginine could form adducts with electrophilic metabolites like the HCAs, especially if they are exposed in a peptide with little or none secondary structure. The doubly-charged species, $[1309.5]^{+2}$, was selected for fragmentation in an LC-MS/MS experiment, and the recorded spectrum is shown in Figure 4. The fragment ions generated, mostly singly charged *b*- and *y*-ions (Biemann 1990), were consistent with the predicted fragmentation pattern for the 20-amino acid peptide. Other ions, y_{11} - y_{14}, b_{12} - b_{13}, and internal fragment 7-12, were present and correspond to the PhIP modification observed from the parent peptide adduct mass. The cysteine was therefore determined to be the site of covalent modification by deduction from the unmodified and modified fragment ions (Figure 4B). Other covalent modifications that have been reported using other metabolic forms of PhIP were not observed in these reactions (Reistad 1994). Additionally, this covalent adduct is different from the sulfinamide linkage observed between serum albumin and IQ, in that the cysteinyl is not oxidized (Turesky 1987).

The intensity of the modified fragment, y_{11}*, *m/z* 1566.5 made it possible to select this ion for further fragmentation to gain more structural detail. A three-stage MS experiment was performed with the same parameters described for the MS/MS of *m/z* 1309.5 with subsequent fragmentation of *m/z* 1566.5 (Figure 5). After loss of water from this daughter ion, the next most abundant ion in the spectrum was a loss of 256 *m/z*

1310.4. This loss may suggest a structure of the amino-sulfur linkage intact, and this type of fragmentation pattern is seen with MS/MS experiments of the reaction products of PhIP and cysteine (data not shown). This further supports the structure of the covalent modification shown in Figure 4. Ultimately the structure we have suggested will require confirmation by 1H-NMR.

This is the first MS/MS characterization of a covalent adduct formed between a peptide or protein and *N*-acetoxy-PhIP. It is proposed that the results herein may be consistent with adduct formation *in vivo* since there is a free cysteine in rat and human serum albumin that is suitably poised for interaction with the HCA. This does not preclude, however, that this is the only adduct formed *in vivo* and studies are underway to test these hypotheses.

Acknowledgments

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Figure Legends

Figure 1. Proposed metabolic activation of PhIP, where **a.** and **b.** correspond to acetyltransferase and sulfotransferase activities, respectively (Buonarati 1990; Boobis 1994).

Figure 2. A. Base peak chromatogram of the peptide VSATWHLQKCPYERMDFNIG in a control reaction. **B.** The average spectrum corresponding to the peak at 15.9 min shown in panel A. The peptide was identified by *m/z* 1198.5. **C**. Base peak chromatogram of *N*-acetoxy-PhIP control reaction. **D**. The average spectrum corresponding to the peaks between 5 and 7 min shown in panel C. The degradation of *N*-acetoxy-PhIP to *N*-hydroxy-PhIP was confirmed by the presence of the singly charged ion, *m/z* 241.2.

Figure 3. A. Base peak chromatogram of peptide-PhIP reaction. The arrow (retention time: 18.9 min) indicates the elution of the peptide-PhIP adduct. **B.** The average spectrum corresponding to the peak at 18.9 min shown in panel A. The adduct was identified with the presence of doubly and triply charged ions, *m/z* = 1309.5 and 873.5, respectively.

Figure 4. A. Spectrum from tandem MS experiment of *m/z* 1309.5. The sequence specific *b*- and *y*-ions and internal fragment ions are assigned above the corresponding peak. Fragment ions with an increased mass of +222.0, the corresponding change that would be represented by a covalent attachment of PhIP, are marked with an asterisk. The spectrum was recorded using an activation amplitude of 28% in the ion trap detector. **B.** Amino acid sequence and predicted fragmentation pattern surrounding the cysteinyl-PhIP adduct. The proposed structure of the PhIP-peptide adduct is shown.

Figure 5. Spectrum from a 3-stage MS experiment of m/z 1309.5 and the y_{11}* ion fragment ion m/z 1566.5. The asterisk denotes the loss of 256 and the corresponding structure is illustrated. The spectrum was recorded using an activation amplitude of 28% and 32%, respectively, in the ion trap.

Figure 1

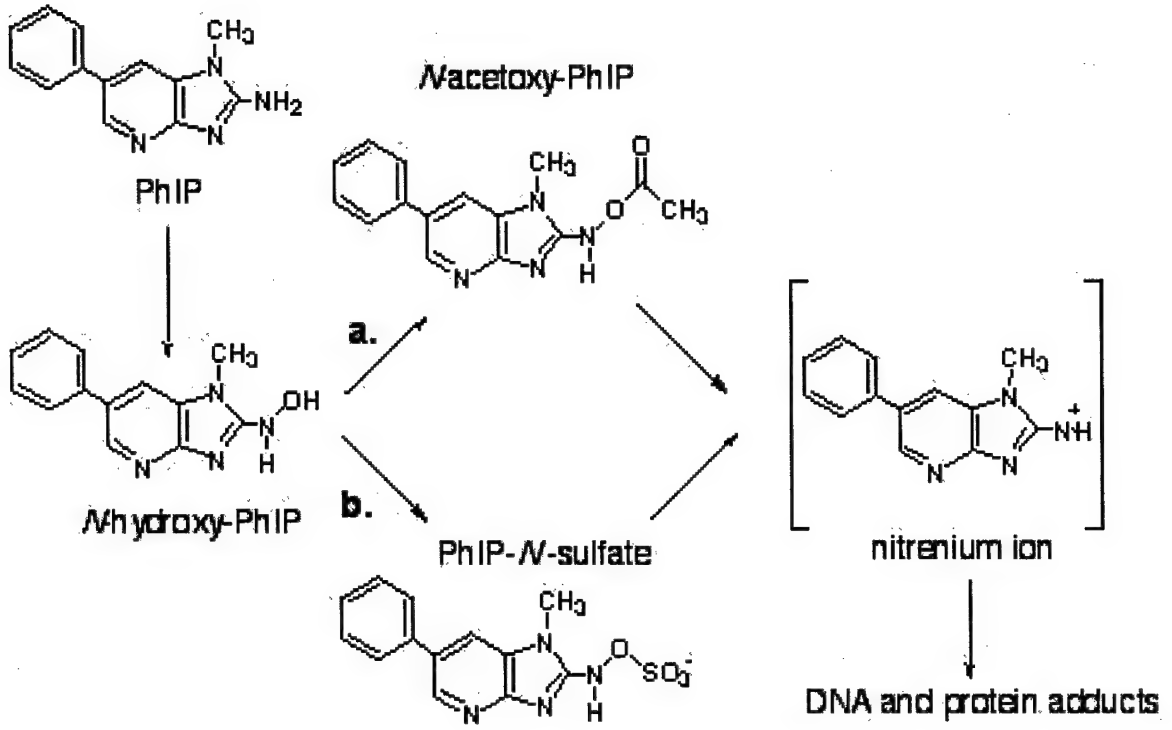

PhIP
N-acetoxy-PhIP
N-hydroxy-PhIP
a.
b.
PhIP-N-sulfate
nitrenium ion
DNA and protein adducts

Figure 2.

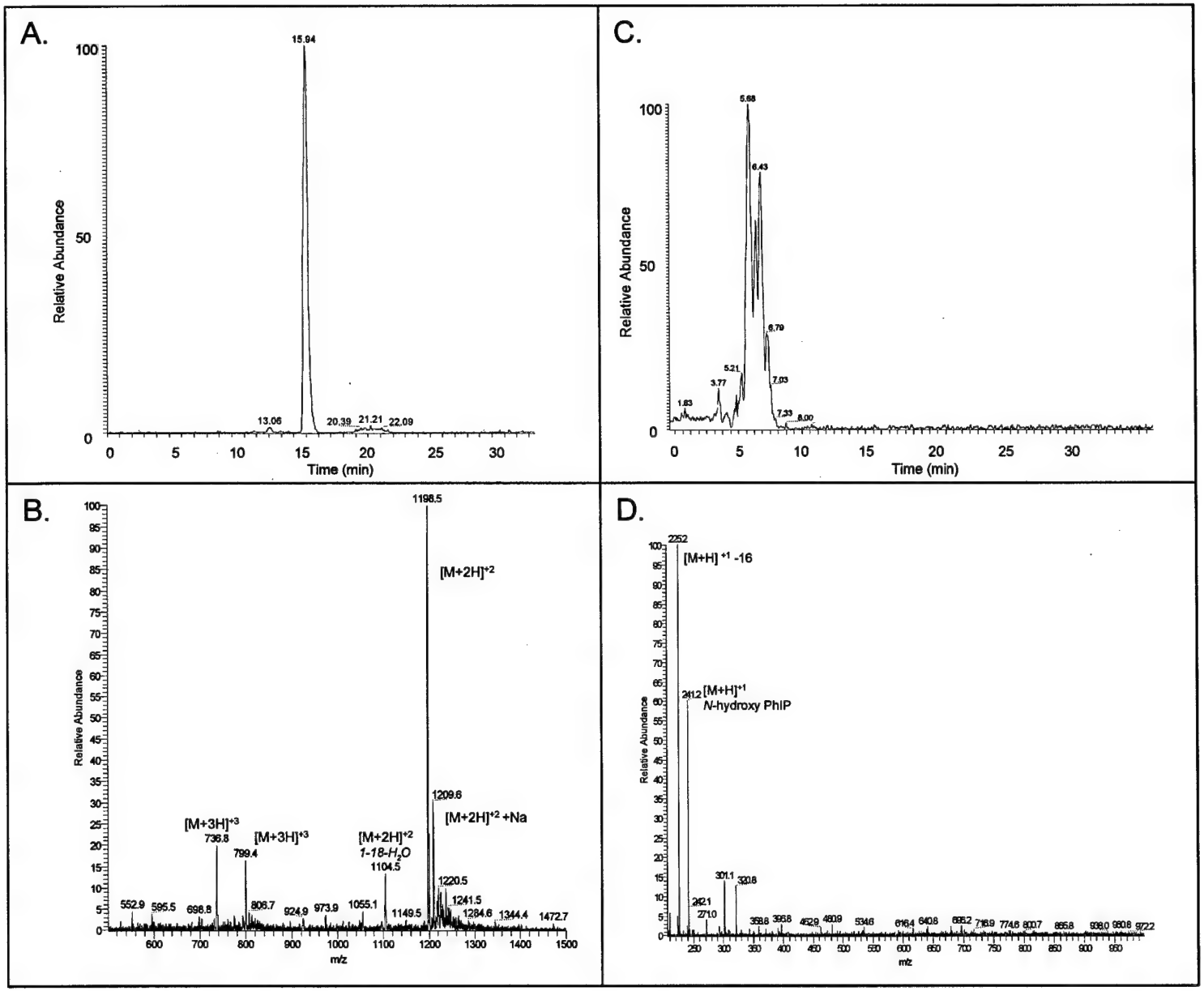

A.
Relative Abundance
Time (min)
15.94
13.06
20.39
21.21
22.09
C.
Relative Abundance
Time (min)
5.68
6.43
6.79
5.21
3.77
7.03
1.83
7.33
8.00
B.
Relative Abundance
m/z
1198.5
[M+2H]+2
1209.6
[M+2H]+2 +Na
[M+3H]+3
736.8
[M+3H]+3
799.4
[M+2H]+2
1-18-H2O
1104.5
1220.5
1241.5
1284.6
1344.4
1472.7
552.9
595.5
698.8
806.7
924.9
973.9
1055.1
1149.5
D.
Relative Abundance
m/z
[M+H] +1 -16
241.2 [M+H]+1
N-hydroxy PhIP
301.1
320.8
242.1
271.0

Figure 3.

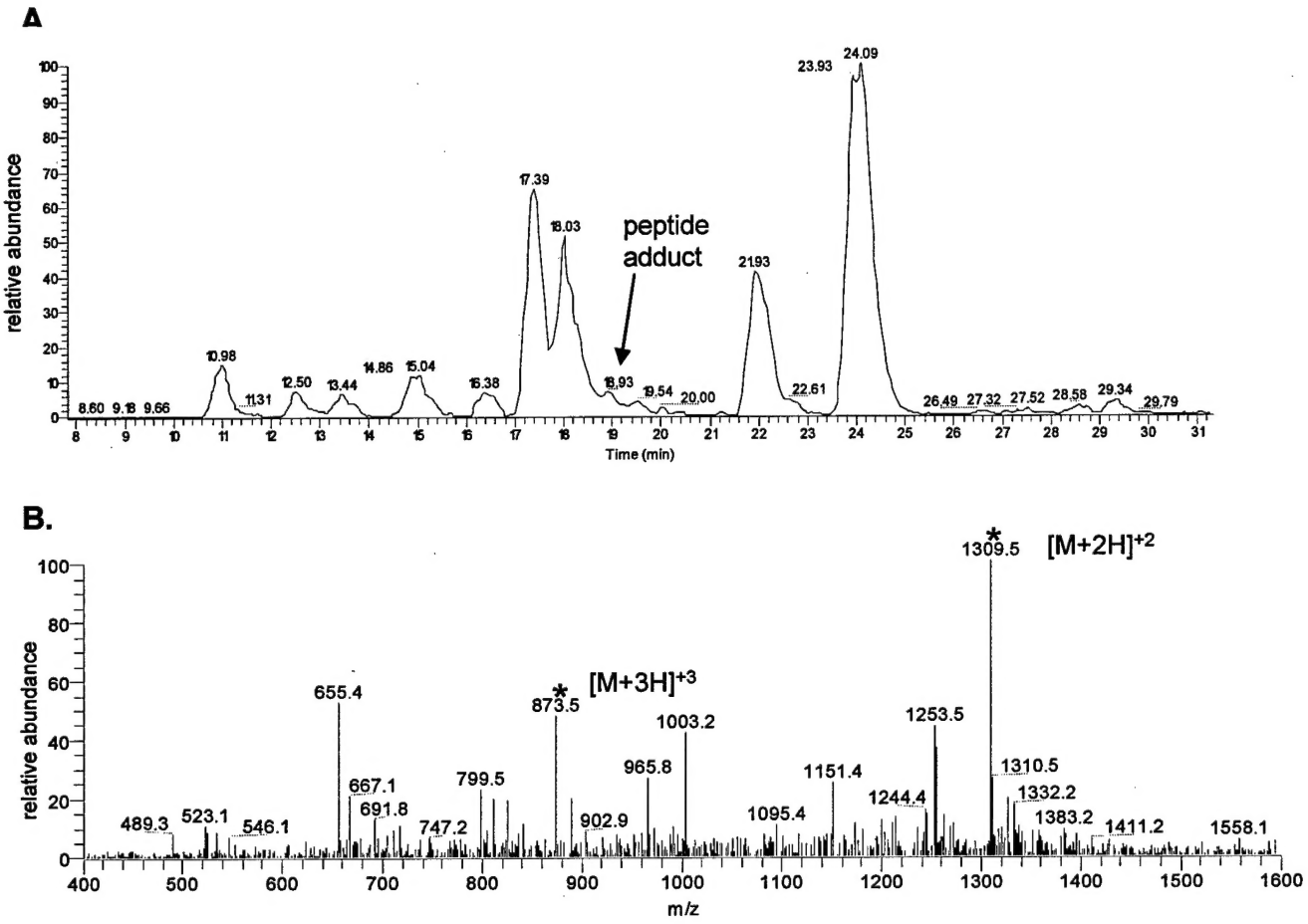

A
relative abundance
Time (min)
peptide
adduct
8.60
9.18
9.66
10.98
11.31
12.50
13.44
14.86
15.04
16.38
17.39
18.03
18.93
19.54
20.00
21.93
22.61
23.93
24.09
26.49
27.32
27.52
28.58
29.34
29.79
B.
relative abundance
m/z
1309.5 [M+2H]+2
873.5 [M+3H]+3
655.4
667.1
691.8
799.5
747.2
902.9
965.8
1003.2
1095.4
1151.4
1244.4
1253.5
1310.5
1332.2
1383.2
1411.2
1558.1
489.3
523.1
546.1

Figure 4 A

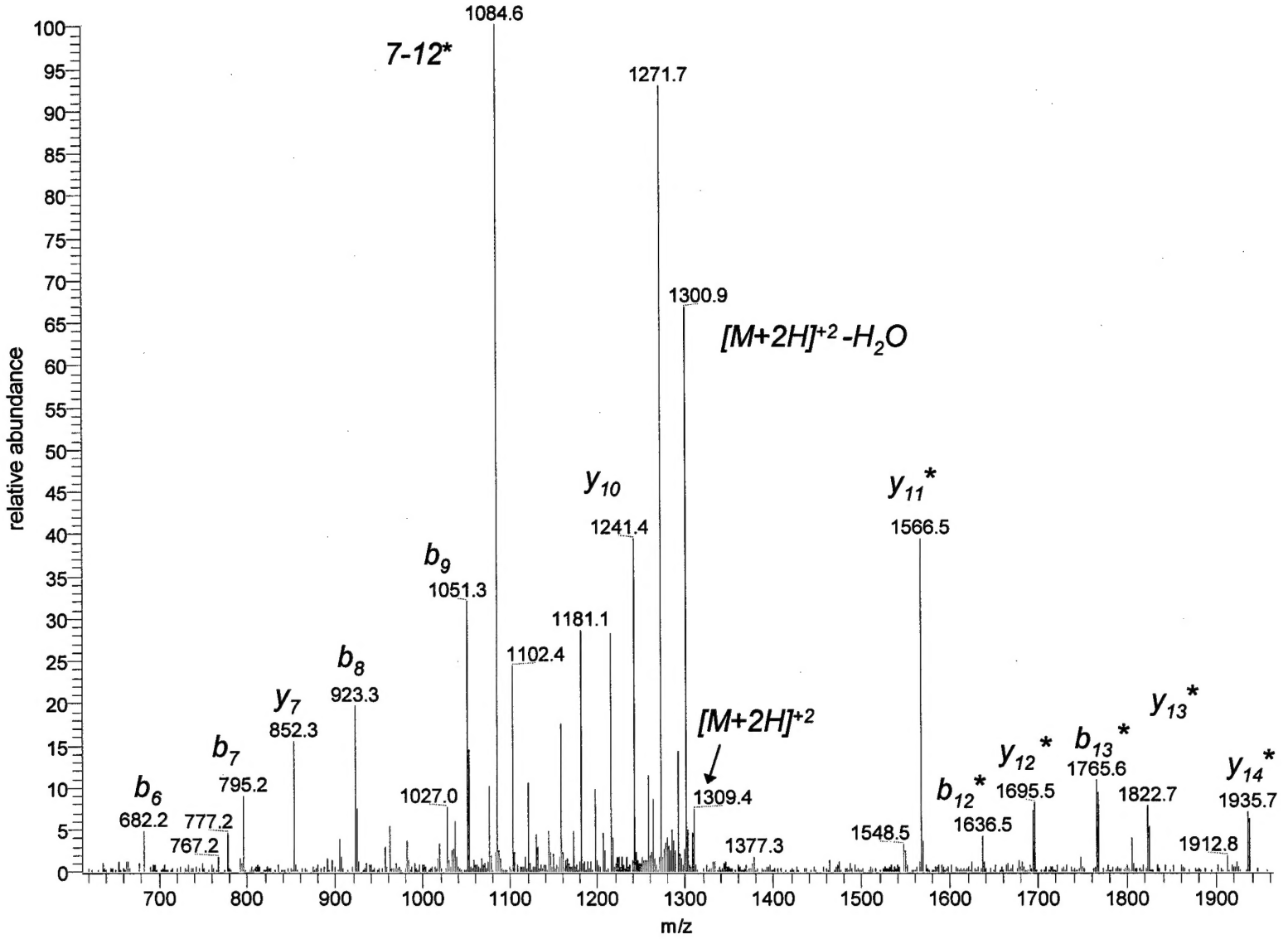

7-12*
1084.6
1271.7
1300.9
[M+2H]+2 -H2O
y10
1241.4
y11*
1566.5
b9
1051.3
1181.1
1102.4
b8
923.3
y7
852.3
b7
795.2
b6
682.2
777.2
767.2
1027.0
[M+2H]+2
1309.4
1377.3
1548.5
b12*
1636.5
y12*
1695.5
b13*
1765.6
y13*
1822.7
y14*
1935.7
1912.8
relative abundance
m/z

Figure 4B

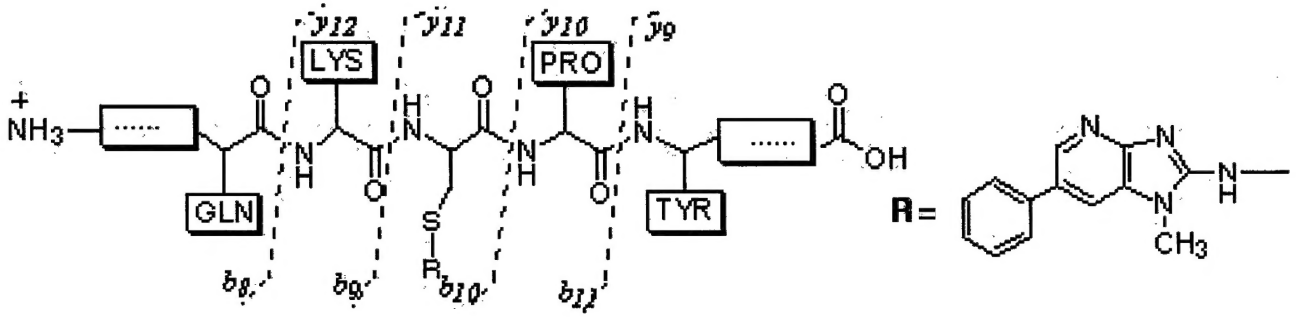
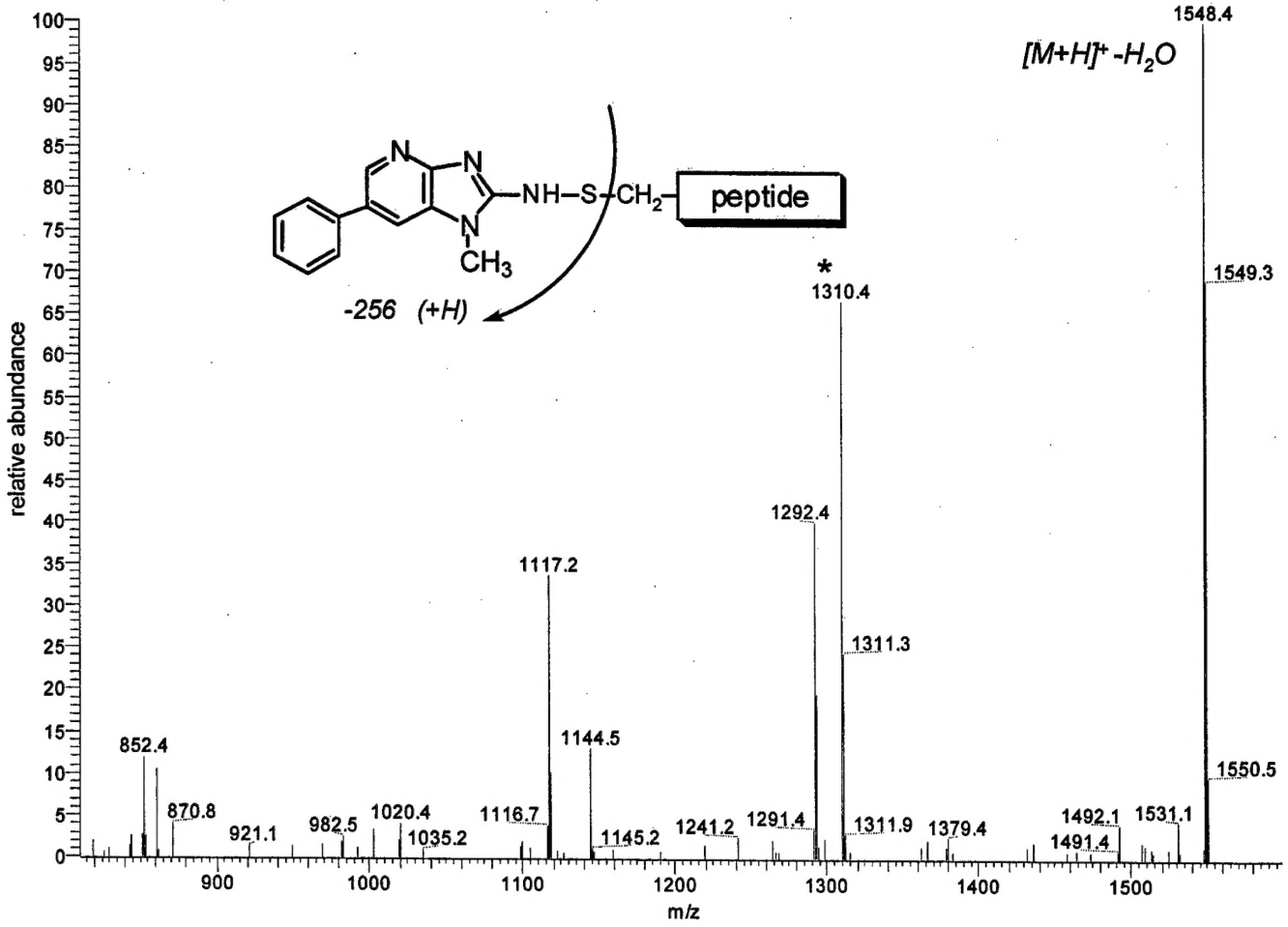


Figure 5


relative abundance
m/z
[M+H]+ -H2O
1548.4
1549.3
1550.5
1310.4
1292.4
1117.2
1311.3
1144.5
852.4
870.8
921.1
982.5
1020.4
1035.2
1116.7
1145.2
1241.2
1291.4
1311.9
1379.4
1491.4
1492.1
1531.1
-256 (+H)
peptide
NH–S–CH2
CH3